Polar delivery of *Legionella* type IV secretion system substrates is essential for virulence

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A recurrent emerging theme is the targeting of proteins to subcellular microdomains within bacterial cells, particularly to the poles. In most cases, it has been assumed that this localization is critical to the protein's function. *Legionella pneumophila* uses a type IVB secretion system (T4BSS) to export a large number of protein substrates into the cytoplasm of host cells. Here we show that the *Legionella* export apparatus is localized to the bacterial poles, as is consistent with many T4SS substrates being retained on the phagosomal membrane adjacent to the poles of the bacterium. More significantly, we were able to demonstrate that polar secretion of substrates is critically required for *Legionella*'s alteration of the host endocytic pathway, an activity required for this pathogen's virulence.

bacterial pathogen | secretion | polar localization | macrophage

For a long time, bacteria were considered to lack subcellular structures and to function simply as vessels for macromolecules such as DNA and protein. With improved technology, specifically better microscopes, an ever-increasing number of proteins have been localized to subdomains within bacterial cells (reviewed in refs. 1–4). The two most notable examples of subcellular domains within rod-shaped bacteria are the midcell and the poles. Because bacteria division occurs in the middle of the cell, cell-division proteins, most notably FtsZ, are spatially restricted to the midcell. Conversely, an even larger number of proteins are localized to the extremities of bacterial cells and are involved in a wide variety of processes including adhesion, motility, chemotaxis, and cell differentiation (4).

In addition to these functions, many specialized secretion systems of Gram-negative pathogens are found at the bacterial poles. These include components of the *Vibrio cholerae* type II secretion system (T2SS), which are restricted to a single pole, resulting in targeted export of substrates from that end of the cell (5). Similarly, the *Salmonella* type III secretion system (T3SS) SPI-2 is found only at the bacterial extremities (6). Although the *Shigella* T3SS was observed to be distributed diffusely over the surface of the cell, the translocon component IpaC was present at only one pole during epithelial cell invasion (7). In addition to these secretion systems, a number of type IV secretion systems (T4SSs) are situated at the bacterial poles. For example, components of a T4SS in *Coxiella burnetii* are polarly localized, as is the *Agrobacterium tumefaciens* VirB T4SS (8–10), although the latter has also been reported to be in helical arrays that extend from the poles (11). Furthermore, many T5SS substrates, including *Shigella flexneri* IcsA, diffusely adherent *Escherichia coli* AIDA-I, and *Bordetella pertussis* BrkA, are confined to a single bacterial pole (12, 13). Moreover, some Gram-positive bacteria also exhibit subcellular localization of their secretion systems. *Streptococcus pyogenes* exports proteins through a single microdomain called the "Ex-Portal" (14), and the *Mycobacterium* T7SS is found at the poles (15, 16).

Therefore, targeted export from specific domains of bacteria is a conserved feature in many Gram-positive and Gram-negative bacteria. Although polar localization of bacterial secretion systems is commonly observed, the significance of this localization remains unconfirmed. Specifically, it is not known whether the poles simply serve as a convenient subdomain for the assembly of multiprotein complexes, whether secretion complexes need to be located at the poles to function properly, or, perhaps more interestingly, whether substrates must be exported from one or both poles.

To address these questions, we focused our attention on the Dot/Icm (defect in organelle trafficking/intracellular multiplication) type IVB secretion system of the pathogenic bacterium *Legionella pneumophila* (17, 18). This remarkable system has been the subject of intense study, because it injects a vast repertoire of effectors, perhaps more than 300 proteins, into the bacterial host cell (reviewed in ref. 19). These T4SS substrates function to prevent phagosome–lysosome fusion and mediate the recruitment of endoplasmic reticulum to the *Legionella*-containing vacuole (LCV), thereby allowing the formation of a unique intracellular compartment in which the bacteria grow. Strikingly, a number of secreted Dot/Icm substrates are retained on the cytoplasmic face of the phagosomal membrane, adjacent to the poles of the bacteria (20–23), suggesting that the *L. pneumophila* Dot/Icm system is located at the bacterial poles. However, it is not known if polar secretion of Dot/Icm substrates is required to mediate survival and replication of *Legionella* within normally bactericidal host cells.

Results

The Dot/Icm T4SS Is Located at the Bacterial Poles. To test the hypothesis that polar secretion is the result of the location of the T4SS, we probed stationary-phase cells using antibodies that recognize several Dot/Icm proteins (DotH, DotG, and DotF) that form part of the *Legionella* T4BSS core complex (24). A Dot-specific signal could be detected at both bacterial poles

Significance

***Legionella pneumophila* is a bacterial pathogen that causes a potentially fatal form of pneumonia (Legionnaires' disease) by replicating within human alveolar macrophages, immune cells that typically kill microorganisms in the lung. *Legionella* avoids intracellular death by injecting many protein effectors into the host cell cytoplasm via a specialized export apparatus called the "Dot/Icm type IVB system." We showed that the Dot/Icm (defect in organelle trafficking/intracellular multiplication) secretion system is restricted to both poles of the bacterium and its localization is a key feature of *L. pneumophila*'s virulence, because nonpolar export of Dot/Icm effectors is ineffectual. Polar export of factors from a specialized secretion system uninvolved with bacterial motility has not previously been shown to be required for the virulence of a bacterial pathogen.**

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in the majority of wild-type *Legionella* cells (Fig. 1*A*). The immunofluorescence signal was specific to each protein, because it was not present in strains lacking the corresponding gene (Fig. 1*A*). Polar localization was not restricted to these Dot/Icm proteins, because bipolar localization of 10 additional Dot/Icm proteins could be detected, as is consistent with previous observations showing polar localization of DotL and IcmW (22) and several epitope-tagged Dot/Icm proteins (25). Our localization of the Dot/Icm proteins was not artificially generated, because we assayed wild-type proteins expressed at native levels from the chromosome. In addition, we did not use GFP fusions, which have been reported sometimes to mislocalize to the poles if overexpressed in bacteria (26). Finally, we were able to detect several control proteins correctly at their natural nonpolar location (Fig. S1), suggesting that the polar localization signal we detected for the Dot/Icm proteins was authentic.

To corroborate the localization of the Dot/Icm proteins using an independent method, we reexamined intact *L. pneumophila* cells by electron cryotomography (ECT) (27, 28). Consistent with our immunofluorescence data, we could observe an electron-dense structure present at each pole between the inner and outer membranes of wild-type cells (Fig. S2 *A–D*). The structure was dependent on the Dot/Icm system, because it was absent in a strain lacking all *dot/icm* genes (Fig. S2*E* and Movie S1). In most cases, we could observe multiple structures at each pole (Fig. S2 and Movie S2), and careful examination of cryotomograms of ~100 poles revealed that *L. pneumophila* contains an average of 4.2 Dot/Icm complexes per pole.

Based on the in vitro localization of the *L. pneumophila* T4SS, we hypothesized that exported Dot/Icm substrates could be detected in the cytoplasm of host cells adjacent to the bacterial poles. To test this theory, we examined the spatial relationship between a Dot protein (DotG) and a secreted substrate (SdeC) within *Legionella*-infected mouse bone marrow-derived macrophages (BMMs). Because our anti-DotG and anti-T4SS substrate antibodies were raised in rabbits, we used a *L. pneumophila* strain expressing DotG fused to an epitope tag. The tagged version of DotG fully complemented a Δ*dotG* mutant for intracellular growth and appropriately colocalized with DotH and DotF within *L. pneumophila* cells (Fig. S3). As predicted, the secreted *L. pneumophila* T4SS substrate SdeC was present in the host cytoplasm adjacent to DotG-HA at both poles of the bacterium (Fig. 1*B*) (21), indicating that polar export of substrates during infection is mediated by the specific localization of the Dot/Icm secretion system.

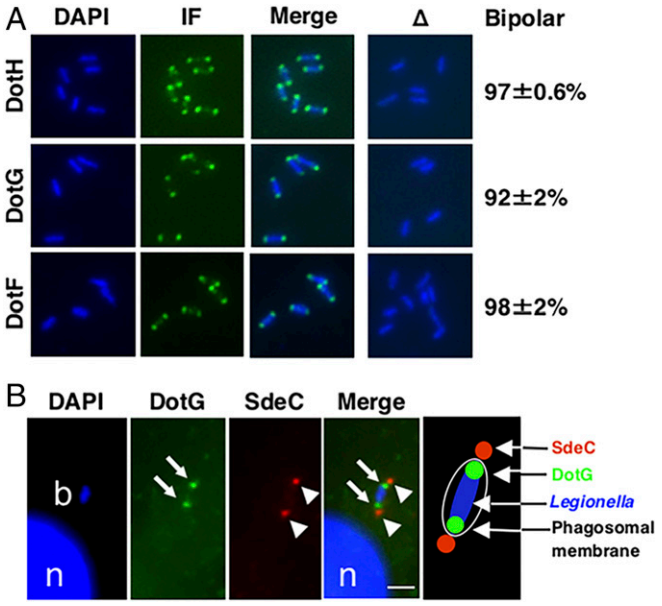

Fig. 1. Bipolar localization of the Dot/Icm T4SS. (*A*) Broth-grown wild-type *L. pneumophila* cells were harvested in stationary phase and stained with antibodies specific to DotH, DotG, or DotF (green) and DAPI (blue). The far right column consists of merged images of the data for the respective *dot* deletions. The percentage of cells having bipolar localization of the Dot/Icm T4SS are shown at the right with the data presented as means ± SEM from three independent experiments in which at least 100 cells were scored in each experiment. (*B*) *L. pneumophila*-infected macrophages were stained with antibodies specific to DotG-HA (green), the T4SS substrate SdeC (red), and DAPI (blue). Arrows indicate the location of DotG, and arrowheads show secreted SdeC. b, bacteria; n, macrophage nucleus. The far right panel shows a schematic of the data shown in the other panels. (Scale bar: 2 μm.)

Polar Localization of T4SS Is Linked with Cell Division. To explore the importance of this localization, we began our investigation by examining how the apparatus is targeted to the bacterial poles. Although DotF localized specifically to both poles of stationary-phase cells (Fig. 1*A*), exponential *L. pneumophila* cells displayed a more complex pattern (Fig. 2 *A–C*). Smaller exponential cells, like stationary-phase cells, had bipolar DotF staining (Fig. 2 *B*, *i* and *ii*), whereas cells that had undergone elongation before division displayed an additional, third focus of DotF in the cell center (Fig. 2 *B*, *iii*). In larger exponential cells that were likely in the process of finishing cell division, two foci of DotF became apparent in the cell center (Fig. 2 *B*, *iv*). Analysis of 200 cells confirmed that shorter cells had two foci of Dot staining, intermediate-sized cells had three foci, and larger cells had four foci (Fig. 2*D*), as is consistent with the notion that initial targeting of DotF occurs at the midcell. Concordant with this idea, we detected Dot/Icm-dependent structures by ECT that were in the bacterial cell envelope near the division plane of dividing cells (Fig. S4 and Movie S3).

Because a newly formed pole of a rod-shaped bacterium is generated from the division septum, the Dot/Icm complex could accomplish polar localization by being targeted to the midcell during cell division or by being targeted to a newly formed pole after cell division has been completed. To confirm that *Legionella* used the former mechanism, we examined T4SS localization in bacterial filaments generated by inhibiting a late cell-division protein, FtsI, that is necessary for the completion of septum formation (29). Treatment of *Legionella* cells with aztreonam, an FtsI inhibitor, generated filaments that do not contain septa between the individual cell units (Fig. S5). Notably, these filaments possessed DotF at periodic points along the filament (Fig. 2*E*), indicating that DotF was being targeted to the future site of division and ruling out the possibility that targeting occurred after the completion of cell division. We were able to observe a similar result for both DotF and DotH using a different FtsI inhibitor, cephalexin (Fig. S5).

The linkage of T4SS localization to cell division suggests that the physical mechanism for targeting may likewise be dependent on the cell-division machinery. To investigate this hypothesis, we examined filaments created by the depletion of FtsZ, the protein responsible for the first committed step in bacterial cell division. In contrast to the FtsI-inhibited filaments, FtsZ depletion generated filaments with defined foci of Dot protein only at the cell poles (Fig. 2*F*). Thus, the Dot/Icm T4SS complex achieves polar localization via a component that is present and/or functions between the action of FtsZ and FtsI during cell division.

Polar Localization Is Critical for *Legionella* Virulence. Because a detailed molecular mechanism by which the Dot/Icm complex targets to the poles is not yet known, we undertook an alternative approach to studying the importance of polar localization by changing the morphology of the cell. Rod-shaped bacterial cells can be converted into spherical cells by exposing them to the small molecule A22 [S-(3,4-dichlorobenzyl) isothiourea], which

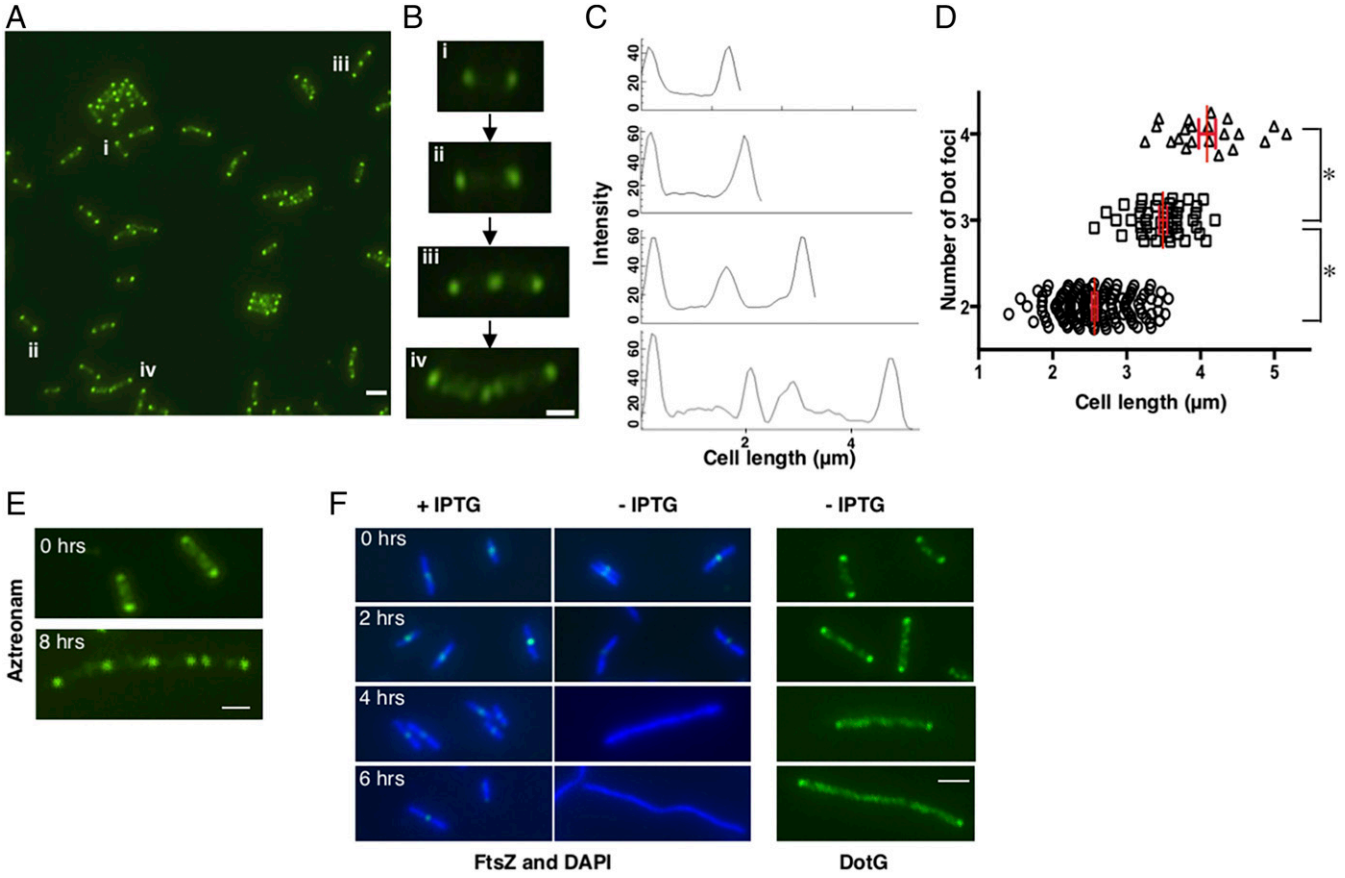

Fig. 2. The Dot/Icm T4SS is targeted to the poles by first localizing to the midcell of exponentially growing cells. (*A*) *L. pneumophila* cells were harvested at exponential-growth phase and were stained with a DotF antibody (green). (*B*) Representative cells of increasing length from *A* are shown under higher magnification. (*C*) ImageJ analysis of fluorescence intensity along the axis of the corresponding cells in *B*. (*D*) Quantitation of the number of Dot foci as a function of cell length. A total of 200 cells were analyzed with ImageJ; data are presented as means ± SEM; asterisks indicate a statistically significant difference ($P < 0.05$) by Student's *t* test. (*E*) Filaments generated by growing *L. pneumophila* in the absence (0 h) or presence (8 h) of the FtsI inhibitor aztreonam were stained with the DotF antibody (green). (*F, Left* and *Center*) *L. pneumophila* filaments generated by depletion of FtsZ. Cells containing isopropyl β-d-1-thiogalactopyranoside (IPTG)-regulated *ftsZ* were grown in the presence (wild type) or absence (FtsZ-depleted) of IPTG and then were stained with an antibody to detect FtsZ (green) and DAPI (blue). (*Right*) FtsZ-depleted cells were stained with an antibody to DotG (green). (Scale bars: 1 μm in *B* and 2 μm in *A*, *E*, and *F*.)

inactivates the bacterial cytoskeletal component MreB (30). As in *E. coli* and *Caulobacter*, A22 treatment of *L. pneumophila* resulted in a mixed population of cells with altered cell morphology. Rod-shaped *L. pneumophila* cells first became amorphous structures, which we referred to as "UFO-shaped cells," before becoming round (Fig. 3). The morphological change of *L. pneumophila* cells by A22 was both dose dependent (Fig. 3*A*) and time dependent (Fig. 3*B*) and resulted in a mixed population consisting of equal numbers of rods, UFOs, and cocci. A22 treatment also had a pronounced effect on the localization of the Dot/Icm apparatus (Fig. 3) when the Dot proteins were completely delocalized in round cells (Fig. 3*C*, *Inset* for DotF). As a result, we were able to alter the natural localization of the *L. pneumophila* T4SS by inhibiting MreB function, similar to the effect seen for the polarly localized *Shigella* IcsA protein (31).

This result allowed us to test if polar localization was important for *L. pneumophila* virulence by examining whether A22-treated cells could properly target the LCV to avoid the endocytic pathway. As previously described (32), the majority of LCVs containing the wild-type *L. pneumophila* strain Lp02 were able to avoid colocalization with the endocytic marker LAMP-1, whereas more than 90% of the LCVs with the *dotA*-deficient strain Lp03 were LAMP-1$^+$ (Fig. 3*D*). A22-treated cells that remained rodlike were still able to prevent phagosome–lysosome fusion at levels equal to untreated cells (only 26% LAMP-1$^+$). In contrast, LCVs containing UFO-shaped cells were significantly defective in the avoidance of LAMP-1 (~60% LAMP-1$^+$), whereas LCVs containing cocci-shaped cells almost always colocalized with LAMP-1 (~90% LAMP-1$^+$) (Fig. 3*D*). Thus, A22 treatment of *L. pneumophila* altered the localization of the Dot/Icm complex and resulted in a dramatic defect in the pathogen's ability to alter the host's endocytic pathway.

Because these results were generated using a drug, it was possible that the change in T4SS localization was not directly responsible for the mistargeting of the LCV. For example, A22 might have had an indirect effect on protein synthesis, perhaps by preventing the expression of T4SS substrates normally expressed in the early stationary phase. We excluded this possibility by demonstrating that A22-treated UFO-shaped cells were able to express a number of T4SS substrates and remained capable of expressing GFP from an inducible promoter (Fig. S6). An alternative explanation for the result of our A22 treatment was that altered cell shape, rather than nonpolar localization of the T4SS, caused the mutant LCV to fail to form a replicative phagosome. Although we currently are unable to eliminate this possibility completely, we do know that long filaments generated by depleting FtsZ are still competent to alter the endocytic pathway of host cells (Fig. S7), implying that cell shape per se is not critical to the intracellular growth of *L. pneumophila* as long as the cells retain polar T4SS complexes.

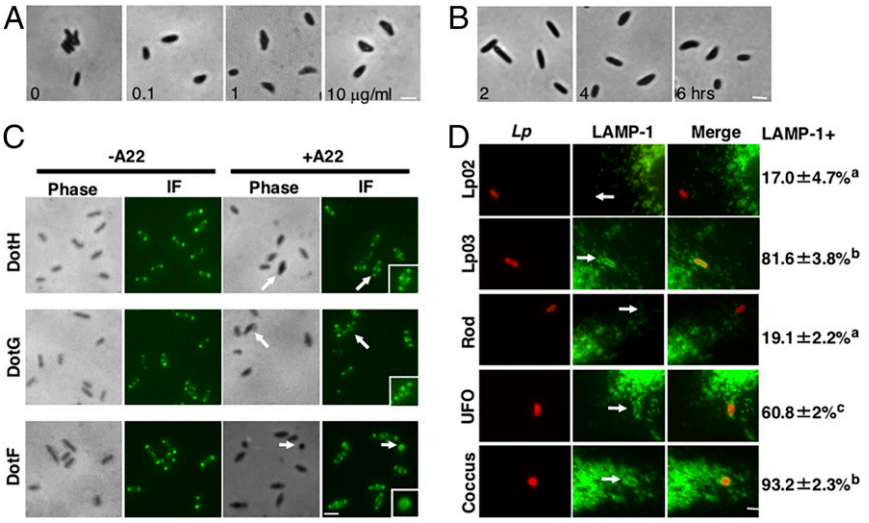

Fig. 3. Bipolar localization of the Dot/Icm T4SS is necessary for alteration of the endocytic pathway by *L. pneumophila*. (*A*) Exponentially growing *L. pneumophila* was treated with a range of the MreB inhibitor A22, followed by 6 h of growth, and then was viewed by phase-contrast microscopy. (*B*) *L. pneumophila* was treated with 1 μg/mL of A22 for 2, 4, or 6 h. (*C*) Bacterial cells were untreated or were treated with A22 and stained with antibodies to DotH, DotG, or DotF. Displayed are phase-contrast (labeled "phase") and immunofluorescence (labeled "IF") images. The arrows indicate representative cells whose images are enlarged in the *Insets*. (*D*) Macrophages were infected with wild-type *L. pneumophila* (Lp02), a *dotA* mutant (Lp03), and A22-treated *L. pneumophila* of various shapes (rod, UFO, and cocci) and were stained with antibodies for *Legionella* (red) and LAMP-1 (green). Arrows indicate the position of the LCV. The percentages of various types of LAMP-1$^+$ cells of are shown to the right and were obtained from three independent experiments, each consisting of 100–200 cells. Data are presented as means ± SEM. Means with different superscript letters differ significantly by one-way ANOVA; $P < 0.05$. (Scale bars: 2 μm.)

Polar Secretion of T4SS Substrates Is Required for Replication Within Host Cells. Based on these results, we hypothesized that LCVs containing cells with nonpolar T4SS complexes failed to avoid the host's endocytic pathway because they were nonfunctional, perhaps because of their improper assembly. To test if these Dot/Icm complexes were unable to secrete proteins, we repeated the macrophage infections using A22-treated cells and simultaneously scored the LCVs for LAMP-1 colocalization and the presence of the exported substrate SdeC. Similar to wild-type cells, the majority of LCVs with rod-shaped *L. pneumophila* cells avoided the endocytic pathway (i.e., were LAMP-1$^-$) and displayed SdeC on their surface (Fig. 4). In contrast, a large number of LCVs with UFO-shaped *L. pneumophila* cells were LAMP-1$^+$, but most were secretion competent (Fig. 4). Even more striking, almost all the LCVs with coccus-shaped bacteria contained SdeC on their surface but were LAMP-1$^+$ (Fig. 4). This phenotype is unprecedented, because LCVs that mistarget into the endocytic pathway (e.g., *dot/icm* mutants) have never been shown to export Dot/Icm substrates (19). The result was not specific to SdeC, because similar results were obtained for two other effectors, LidA and SidC (Fig. S8 *A* and *B*), and in each case we did not detect a significant decrease in the amount of exported substrate (Fig. S8*C*). Proper translocation of the T4SS substrates into the host cytoplasm, rather than aberrant secretion of protein into the lumen of the LCV, was confirmed by differential permeabilization using methanol (Fig. S9). Moreover, the

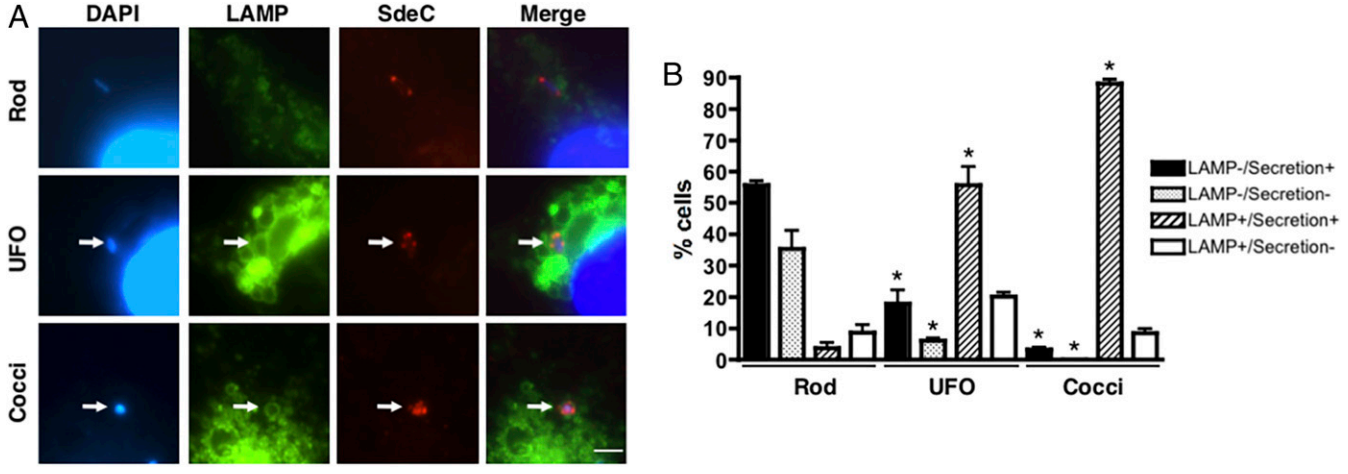

Fig. 4. Polar export of T4SS substrates is required for the proper targeting of the LCV. (*A*) Macrophages infected with *L. pneumophila* having a modified cell shape were fixed and stained with antibodies to LAMP-1 (green) and the secreted effector SdeC (red) or DAPI (blue). Shown are a LAMP$^-$/secretion$^+$ rod-shaped cell and LAMP$^+$/secretion$^+$ UFO-shaped and round cells. (*B*) Phagosomes were scored for colocalization with LAMP-1 and/or secreted SdeC. Results are shown for three independent experiments; at least 75 cells of different shapes were scored in each experiment. Data are presented as means ± SEM. Asterisks indicate a statistical difference ($P < 0.05$) of each category of UFO-shaped or cocci cells compared with rod cells by Student's *t* test. (Scale bar: 2 μm.)

result was not caused by an indirect effect of the drug because it could be phenocopied using *L. pneumophila* cells lacking *bolA*, a gene involved in cell shape in Gram-negative bacteria (Fig. S10). Thus, polar secretion of T4SS substrates is absolutely required for this pathogen to replicate within host cells.

Discussion

Based on previous observations that a number of Dot/Icm-secreted effectors localize to the cytoplasmic face of the LCV adjacent to the bacterial poles, we hypothesized and then showed that components of the Dot/Icm T4SS localize to both bacterial poles and also demonstrated that the substrate SdeC was exported juxtaposed to the poles of the bacterium during an infection. Although a detailed mechanism by which Dot/Icm is localized to the cell termini remains to be elucidated, the localization appears to be initiated by targeting components of the T4SS to the bacterial midcell during cell division. Finally, and most significantly, we demonstrated that altering the shape of *Legionella* cells resulted in nonpolar secretion of Dot/Icm effectors that was insufficient to alter the host's endocytic pathway, thereby showing that polar secretion of substrates is required for *Legionella*'s virulence.

A number of mechanisms have been proposed to explain how bacteria target proteins to their poles (2, 4). The most common mechanism is based on a "diffusion and capture" mechanism whereby proteins diffuse within the cell, encounter a factor that is already at the pole, and then become trapped at that location. Proteins that are independently targeted to the pole have been described as "anchor" or "landmark" proteins, and their localization can occur by several different mechanisms. Some proteins can exploit unique features of the poles of rod-shaped bacteria, including enhanced negative curvature, altered lipid composition, and/or a more stable peptidoglycan, to be localized directly to those subcellular domains (4). For example, *Bacillus subtilis* DivIVA localizes to the poles via its specific recognition of the increased negative curvature of the membrane (33). Similarly, the transmembrane *E. coli* protein ProP targets to the bacterial ends via its affinity for the anionic phospholipid cardiolipin, which is enriched at the extremities of the bacterial cell (34). A second strategy for polar localization, exhibited by some chemoreceptor arrays, is based on their ability to self-assemble into large complexes; this self-assembly can occur spontaneously at sites distant from the midcell, likely because of nucleoid exclusion (35). A third strategy involves exploitation of the cell-division machinery, because the division site of rod-shaped bacteria eventually matures into two new poles. *B. subtilis* DivIVA also localizes to division septa because of their concave membrane curvature and then can recruit other proteins to the site of cell division and future poles (33, 36).

In the case of the *L. pneumophila* Dot/Icm system, we prefer the hypothesis that it is localized to the cell poles based on some variation of the third mechanism. Although we observed the Dot/Icm system primarily at both poles of stationary-phase *L. pneumophila* cells, we could detect Dot/Icm components and a Dot/Icm-dependent structure at newly formed septa of exponentially grown cells. Based on inhibition studies, we discovered that proper localization of this T4SS was dependent on a factor that acts between FtsZ and FtsI during cell division. Therefore we favor the idea that *Legionella* uses a cell-division protein as an initial anchor to target the Dot/Icm proteins to the poles.

Interestingly, our ECT analysis of intact bacterial cells revealed that on average *L. pneumophila* contains only approximately four Dot/Icm structures at each pole. The limited number of secretion complexes was somewhat surprising, given that *L. pneumophila* is known to export ~300 Dot/Icm substrates (19), and suggests a need for a large number of secretion apparatus per cell. However, the Dot/Icm substrates are predicted to function as toxins within the host cell and are not anticipated to be expressed at high levels. Consistent with this notion, it was previously determined that each *L. pneumophila* cell delivers only ~200 molecules of the Dot/Icm substrate VipD into an infected host cell (37). Therefore it is possible that each *L. pneumophila* cell may need to be capable of exporting fewer than ~60,000 molecules (300 effectors × 200 molecules per cell). Considering that a single *Salmonella* SPI-1 T3SS can export ~6,000 molecules of a single effector (SipA) (38) and a single flagellum can export ~20,000 flagellin molecules (39), it is feasible that a small number of Dot/Icm complexes are sufficient for translocation of the vast arsenal of T4SS effectors used by *L. pneumophila*.

Even more surprising than the limited number of Dot/Icm complexes per cell was the observation that the nonpolar localized Dot/Icm apparatus were still able to secrete effectors into the host cell, because we had assumed that polar localization of the Dot/Icm system would be required for its proper assembly and activity. However, we discovered that nonpolar export of Dot/Icm effectors was insufficient to prevent the LCV from aberrantly entering into the endocytic pathway. To our knowledge, a requirement for polar secretion has not previously been demonstrated for a specialized secretion system. There are a number of possible explanations why polar secretion may be advantageous to *L. pneumophila*. For example, polar secretion of substrates could result in an increased localized concentration of certain effectors near the LCV, and such localization might be required to achieve a critical threshold concentration necessary for their activity. Alternatively, targeted secretion could mediate a hierarchical display of effectors on the LCV, perhaps using Dot/Icm substrates initially anchored to the LCV membrane via their propensity to bind phosphatidylinositol 4-phosphate [PI(4)P]-enriched membranes (e.g., SidM/DrrA, SidC, and SdcA) (40). Finally, the spatiotemporal properties of the LCV may have conferred a steric restriction whereby functional secretion occurred only at the bacterial poles. Because the newly formed LCV resembles a macropinosome (41), initial contacts between *L. pneumophila* and the LCV membrane in such a "spacious phagosome" would occur primarily at the bacterial poles, thereby providing an evolutionary advantage to polar localization of the secretion system. These concepts are not mutually exclusive, and additional investigation will be required to elucidate their role in determining why polar secretion is important to *L. pneumophila* virulence. Furthermore, similar studies in other pathogens will likely be informative regarding the overall importance of polarized bacterial secretion systems.

In conclusion, we have shown that the *L. pneumophila* Dot/Icm T4SS is present at both poles of each bacterial cell and that the localization of a specialized secretion system to a discrete subcellular domain is essential for the virulence of a bacterial pathogen. Future experiments examining how this large macromolecular complex accomplishes polar localization, presumably by exploiting a component of the cell-division machinery, will likely provide useful information about how other proteins are targeted to the poles. Finally, further analysis of polar secretion will likely reveal key insights about how *L. pneumophila* manipulates host cell functions.

Experimental Procedures

Strains and Media. *L. pneumophila* strains were cultured in *N*-(2-Acetamido)-2-aminoethanesulfonic acid (ACES) yeast extract (AYE) broth or on buffered charcoal yeast extract (CYE) plates. All strains were constructed in Lp02, a thymidine auxotroph of the *L. pneumophila* Philadelphia-I strain (42). The media were supplemented with 100 μg/mL thymidine as needed (AYET, CYET). All media and antibiotic concentrations for *E. coli* and *L. pneumophila* were used as previously described (43).

Immunofluorescence Microscopy of *Legionella*. *L. pneumophila* proteins were localized by modification of an immunofluorescence procedure previously described for *E. coli* (44). In brief, 7 μL of a culture of *L. pneumophila* were fixed for 5 min in 200 μL of 80% methanol, washed in PBS, and allowed to adhere to poly-L-lysine–coated microscope slides. A lysozyme solution [3 mg lysozyme/mL in 25 mM Tris·HCl (pH 8.0), 50 mM glucose, 10 mM EDTA] was

used to permeabilize cells. The permeabilized cells then were incubated with primary antibodies followed by decoration with Oregon Green-conjugated goat anti-rabbit IgG. The cells were also stained with DAPI to detect DNA and then were viewed using an Olympus fluorescence microscope (100× objective). Measurements of fluorescence intensity and cell length were done using ImageJ (National Institutes of Health).

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